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PATENT ABSTRACTS OF JAPAN

(11)Publication number : **06-277067**

(43)Date of publication of application : **04.10.1994**

(51)Int.CI.

C12N 15/53
C12N 1/21
C12N 9/04
//(C12N 15/53
C12R 1:13)
(C12N 1/21
C12R 1:13)

(21)Application number : **05-068153**

(22)Date of filing : **26.03.1993**

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(54) GENE DNA CODING ACETOHYDROXY ACID ISOMEROREDUCTASE

(57)Abstract:

PURPOSE: To produce L-isoleucine or L-valine in high efficiency by using a gene originated from coryneform bacteria and coding acetohydroxy acid isomeroreductase.

CONSTITUTION: A gene coding acetohydroxy acid isomeroreductase and consisting of 1014 base pairs coding 338 amino acids is separated from Brevibacterium flavum MJ-233 and introduced into the same kind of coryneform bacteria. L-isoleucine or L-valine can be produced in high efficiency from a new viewpoint by using the transformed coryneform bacteria.

LEGAL STATUS

[Date of request for examination]

[Date of sending the examiner's decision of rejection]

[Kind of final disposal of application other than the examiner's decision of rejection or application converted registration]

[Date of final disposal for application]

[Patent number]

[Date of registration]

[Number of appeal against examiner's decision of rejection]

[Date of requesting appeal against

examiner's decision of rejection]
[Date of extinction of right]

[Detailed Description of the Invention]
[0001]
[Industrial Application] this invention relates to the coryneform group of bacteria by which the transformation was carried out by the recombination plasmid containing the gene DNA which carries out the code of the aceto hydroxy-acid iso melodrama reductase (E. C.1.1.1.86) of the coryneform-group-of-bacteria origin, and this gene, and this plasmid.

[0002]
[Description of the Prior Art] The aceto hydroxy-acid iso melodrama reductase (E. C.1.1.1.86) As one of the biosynthesis genes of an isoleucine and a valine You may set to Escherichia coli (Escherichia coli), and it inquires. [Journal of Biological Chemistry (Journal of Biological Chemistry) 261, 2441-2450, 1986], In addition, the SHINEKO cis-teeth (Synechocystis sp.) origin gene [journal OBU bacteriology (Journal ofBacteriology) 174, 7910-7918, 1992], The RAKUTOKOKKASU RAKUTISU (Lactococcus lactis) origin gene [journal OBU bacteriology (Journal of Bacteriology) 174, 6580-6589, 1992], The rhizobium MERIRO tea (Rhizobium meliloti) origin gene [journal OBU bacteriology (Journal of Bacteriology) 173, 7756-7764, 1991], The Saccharomyces cervisiae (Saccharomyces cerevisiae) origin gene [NUKUREIKKU acid research (Nucleic Acid Ressearch) 14, 9631-9651, 1986], The primary structure of the spinner thia ORERASEA (Spinacia oleracea) origin gene [biochemical journal (Biochemiacl Journal) 277,496-475 and 1991] is determined. . However, there is no conventional example of a report about the primary structure of the aceto hydroxy-acid iso melodrama reductase (E. C.1.1.1.86) gene of the coryneform-group-of-bacteria origin which is important bacteria on industry.
[0003]
[Problem(s) to be Solved by the Invention] The purpose of this invention is isolating the gene which carries out the code of the aceto hydroxy-acid iso melodrama reductase (E. C.1.1.1.86) of the coryneform-group-of-bacteria origin, introducing this gene into a coryneform group of bacteria of the same kind, and manufacturing L-isoleucine or L-valine efficiently from a new viewpoint using this coryneform group of bacteria.
[0004]
[Means for Solving the Problem] As a result of repeating research wholeheartedly that the above-mentioned purpose should be attained, this invention persons succeed in isolating an aceto hydroxy-acid iso melodrama reductase gene from a coryneform-group-of-bacteria chromosome, and came to complete this invention. According to this invention (1) in this way Gene DNA which carries out the code of the aceto hydroxy-acid iso melodrama reductase of the coryneform-group-of-bacteria origin (2) Recombination plasmid into which this gene DNA was introduced (3) Coryneform-group-of-bacteria ** by which the transformation was carried out by this recombination plasmid is offered.
[0005] Hereafter, this invention is further explained to a detail. The "gene DNA which carries out the code of aceto hydroxy-acid iso melodrama Jupiter-VIII KUTAZE" of this invention means the enzyme DNA which compounds 2 and 3-dihydroxy isovaleric acid from the enzyme which compounds a 2 and 3-dihydroxy-3-methyl valeric acid from 2-aceto-2-hydroxybutyric acid, or 2-acetolactic acid, i.e., the gene which carries out the code of aceto hydroxy-acid iso melodrama Jupiter-VIII KUTAZE (E. C.1.1.86).
[0006] Although compounding is also possible after the base sequence is determined, the DNA fragment (this may be hereafter called "A fragment" for short) containing the gene

which carries out the code of aceto hydroxy-acid iso melodrama Jupiter-VIII KUTAZE As a microorganism which can carry out cloning from the microorganism which generally has aceto hydroxy-acid iso melodrama Jupiter-VIII KUTAZE productivity, and serves as the source of supply a coryneform group of bacteria -- Brevibacterium flavum (Brevibacterium flavum) MJ-233 (FERM BP-1497) and its origin stock are used especially advantageously

[0007] :A fragment which will be as follows if an example of the fundamental operation for preparing A fragment from these source-of-supply microorganisms is described exists on the chromosome of the above-mentioned coryneform group of bacteria, for example, Brevibacterium flavum MJ-233 (FERM BP-1497) stock, and can be separated and acquired by the method described below out of the cutting fragment produced by cutting this chromosome by the suitable restriction enzyme.

[0008] First, Chromosome DNA is extracted from a Brevibacterium flavum MJ-233 stock culture. It is a suitable restriction enzyme, for example, Bgl II, about this chromosome DNA. And Chromosome DNA is completely decomposed using EcoRI. A transformation stock is acquired by inserting the DNA fragment obtained in the expression vector 233-3 (Pharmacia manufacture) in Escherichia coli, for example, pKK, after flush-end processing, carrying out the transformation of the isoleucine and valine demand nature Escherichia coli variant Escherichia coli (Escherichia coli) ME 8315 [the National Institute of Genetics, heredity experiment living thing preservation research center;Postcode411, and 1111, Yata, Mishima-shi, Shizuoka-ken] to which the aceto hydroxy-acid iso melodrama Jupiter-VIII KUTAZE gene suffered a loss using this vector, and ****(ing) to a selective medium. Plasmid DNA can be extracted from the transformation stock obtained, and A fragment of the Brevibacterium flavum MJ-233-stock chromosome origin inserted by analyzing by the restriction enzyme can be checked and acquired.

[0009] A fragment obtained in this way is cut using a still more suitable restriction enzyme, the DNA fragment obtained is inserted in vector bra SUMIDO which can be reproduced by Escherichia coli, this vector plasmid is introduced into the aforementioned isoleucine and a valine demand nature Escherichia coli variant by the transformation by the transformation method usually used, for example, a calcium chloride method, the electric pulse method, etc., and it **** to a selective medium.

[0010] Inserted A fragment of the Brevibacterium flavum MJ-233-stock chromosome origin can be checked and acquired by extracting plasmid DNA and analyzing by the restriction enzyme from the transformant obtained. Thus, one of the A fragments obtained starts the above-mentioned Brevibacterium flavum MJ-233 stock chromosome DNA by full decomposition of a restriction enzyme EcoRI, and it is a restriction enzyme Bgl II about it further. The size obtained can mention the DNA fragment of about 2.1 kb(s) by cutting.

[0011] The number of recognition sites when cutting the DNA fragment containing the gene which carries out the code of this aceto hydroxy-acid iso melodrama reductase of about 2.1 kb(s) by various kinds of restriction enzymes, and the size of a cutting fragment are shown in the following table 1.

[0012]

[Table 1]

Table 1 Restriction enzyme The number of recognition sites The size of the piece of cutting (kb) PvuII 1 1.55 0.55 SalI 2 0.95 0.65 0.5 EcoRV 1 1.1 1.0 StuI 1 1.8 0.3 DraI 1

1.4 0.7 [0013] In addition, in this specification, the "number of recognition sites" by the restriction enzyme carried out full decomposition of a DNA fragment or the plasmid under existence of a restriction enzyme, and presented agarose gel electrophoresis and 5% polyacrylamide gel electrophoresis with those decomposition products 1% according to the method of itself known, and the value determined from the number of separable fragments was used for it.
[0014] Moreover, "the size of a cutting fragment" and the size of a plasmid In using agarose gel electrophoresis, it bases on the standard line drawn by the migration distance on the same agarose gel of the DNA fragment of molecular weight known which cuts DNA of the lambda phage (lambda phage) of Escherichia coli by the restriction enzyme Hind III, and is obtained. In using polyacrylamide gel electrophoresis It is based on the standard line drawn by the migration distance on the same polyacrylamide gel of the DNA fragment of molecular weight known which cuts DNA of FAI X 174 phage (phix174 phage) of Escherichia coli by the restriction enzyme Hae III, and is obtained. The size of each DNA fragment of a cutting DNA fragment or a plasmid is computed. The size of a plasmid adds and asks for the size of each cutting fragment. In addition, in the determination of the size of each DNA fragment, the result obtained by agarose gel electrophoresis 1% about the size of the fragment of 1 or more kbs was adopted, and the result obtained from about 0.1 kb(s) by polyacrylamide gel electrophoresis 4% about the size of the fragment of less than 1 kb was adopted.
[0015] On the other hand, the above-mentioned chromosome DNA of Brevibacterium flavum MJ-233 A restriction enzyme EcoRI Bgl II The size obtained by cutting about the DNA fragment of about 2.1 kb(s) the dideoxy-in the base sequence nucleotide enzymatic process (the dideoxy chain termination method --) using a plasmid pUC118 and/or pUC119 (TAKARA SHUZO make) Sanger, F.et.al., and Proc.Natl.Acad.Sci. -- USA74 and p -- 5463 and 1977 can determine Thus, the array number of the array table which carries out the postscript of the gene which carries out the code of the aceto hydroxy-acid iso melodrama reductase determined from existence of the open reading frame of the base sequence of the DNA fragment of the determined above-mentioned abbreviation 2.1kb: It has the array shown in 1 and consists of 1014 base pairs which carry out the code of the 338 amino acid.
[0016] The above-mentioned array number of the after-mentioned array table: The DNA fragment containing the gene which carries out the code of the aceto hydroxy-acid iso melodrama reductase of this invention which includes the base sequence shown in 1 and changes may be compounded not only using what was separated from the natural coryneform-group-of-bacteria chromosome DNA but using the DNA synthesizer usually used, for example, the 394 DNA/RNA synthesizer by the applied biotechnology systems company.
[0017] Moreover, the DNA fragment of this invention acquired from the chromosome DNA of Brevibacterium flavum MJ-233 like the above Unless the function which carries out the code of the aceto hydroxy-acid iso melodrama reductase is spoiled substantially Some bases of a base sequence may be replaced by other bases, or it may be deleted. or the thing to which the base may newly be inserted in and transposition of a part of base sequence is carried out further -- you may be -- any of these derivatives -- although -- it is included by the DNA fragment containing the gene which carries out the code of the aceto hydroxy-acid iso melodrama reductase of this invention

[0018] The size explained in full detail above shows the restriction enzyme cutting spot map of the DNA fragment of about 2.1 kb(s) to drawing 1 . The DNA fragment (A fragment) containing the gene which carries out the code of the aceto hydroxy-acid iso melodrama reductase of this invention can obtain the recombination plasmid in which the high manifestation of the aceto hydroxy-acid iso melodrama reductase is possible within a coryneform group of bacteria by introducing into a suitable plasmid vector, for example, the plasmid vector which contains at least the gene which manages the duplicate proliferation function of a plasmid within a coryneform group of bacteria.
[0019] Moreover, although he can be promotor of a gene own [this] which a coryneform group of bacteria holds, as long as the promotor for making the gene which carries out the code of the aceto hydroxy-acid iso melodrama reductase of this invention discover is the base sequence of the procaryote origin for not being restricted to it and making the imprint of an aceto hydroxy-acid iso melodrama reductase gene start, he may be what promotor.
[0020] As a plasmid vector which contains at least the gene which manages the duplicate proliferation function within a coryneform group of bacteria which can introduce A fragment of this invention For example, the plasmid pCRY30 given in JP,3-210184,A; The plasmid pCRY21 given in JP,2-276575,A, pCRY2KE, pCRY2KX, pCRY31, pCRY3KE And pCRY3KX; the plasmid pCRY2 given in JP,1-191686,A, and pCRY3; -- pAM330; given in JP,58-67679,A -- pHM1519; given in JP,58-77895,A -- pAJ655 [given in JP,58-192900,A] -- pAJ611 and pAJ1844; -- pCG1; given in JP,57-134500,A -- pCG2; given in JP,58-35197,A -- pCG4 and the pCG11 grade of a publication can be mentioned to JP,57-183799,A
[0021] what has the gene which manages the duplicate proliferation function of a plasmid within a coryneform group of bacteria, and the gene which manages the stabilization function of a plasmid within a coryneform group of bacteria as a plasmid vector used by the host-vector system of a coryneform group of bacteria especially -- desirable -- for example, the plasmids pCRY30, pCRY21, and pCRY2 -- KE and pCRY2 -- KE and pCRY2 -- KX, pCRY31, pCRY3KE, pCRY3KX, etc. are used suitably
[0022] As a method of preparing the above-mentioned plasmid vector pCRY30 Plasmid pBY503(about detail of this plasmid, it is referring to JP,1-95785,A) DNA is extracted from BUREBI bacterium suitor thio varnish (Brevibacterium stationis) IFO12144 (FERM BP-2515). The DNA fragment in which a size contains the gene which manages the duplicate proliferation function of the plasmid of about 4.0 kb(s) by the restriction enzyme XhoI (this may be called "duplicate field" below) It starts and the DNA fragment (this may be called "stabilization field" below) containing the gene in which a size manages the stabilization function of the plasmid of about 2.1 kb(s) by restriction enzymes EcoRI and KpnI is started. A plasmid vector pCRY30 can be prepared by including these pieces of bisection in EcoRI, KpnI part, and SalI part of a plasmid pHSG298 (TAKARA SHUZO make).
[0023] Next, introduction of A fragment of this invention to the above-mentioned plasmid vector can cleave the restriction enzyme part which exists only one place for example, in a plasmid vector by this restriction enzyme, and can be performed by processing the aforementioned A fragment and the plasmid vector which clove by S1 nuclease there if needed, and considering as a flush end, or making it connect with the bottom of existence of the suitable adapter DNA by DNA ligase processing.
[0024] Introduction of A fragment of this invention to a plasmid pCRY30 can make a

plasmid pCRY30 able to cleave by the restriction enzyme EcoRI, and can be performed by making the DNA fragment (A fragment) containing the gene which carries out the code of aforementioned aceto hydroxy-acid iso melodrama Jupiter-VIII KUTAZE there connect by the DNA ligase. Thus, the size of this invention introduced and rearranged A fragment of about 2.1 kb(s) to the plasmid pCRY30 developed, and this invention persons named the plasmid plasmid pCRY30-IR. The after-mentioned example explains the detail of the creation method of plasmid pCRY30-IR.

[0025] It becomes possible to introduce into a host microorganism the plasmid in which duplicate proliferation is possible within the coryneform group of bacteria containing the gene which carries out the code of the aceto hydroxy-acid iso melodrama reductase developed in this way, and to produce L-isoleucine and L-valine efficiently stably using the culture of this microorganism. As a host microorganism which can carry out a transformation by the plasmid by this invention, a coryneform group of bacteria (FERM BP-1497), Brevibacterium flavum MJ-233 [for example,], Brevibacterium flavum MJ-233-AB-41 (FERM BP-1498), Brevibacterium flavum MJ-233-ABT-11 (FERM BP-1500), Brevibacterium flavum MJ-233-ABD-21 (FERM BP-1499), etc. are mentioned.

[0026] In addition, above FERM The strain of BP-1498 is FERM. It is the ethanol utilization nature microorganism to which DL-alpha-aminobutyric-acid resistance was positively given by using strain of BP-1497 as an old stock (refer to JP,59-28398,B 3rd - 4 Dutch). Moreover, FERM The strain of BP-1500 is FERM. It is L-alpha-aminobutyric-acid transaminase quantity activity variant which used strain of BP-1497 as the old stock (refer to JP,62-51998,A). Furthermore, FERM The strain of BP-1499 is D-alpha-aminobutyric-acid deaminase quantity activity variant which used strain of FERMBP-1497 as the old stock (refer to JP,61-177993,A).

[0027] Besides these microorganisms BUREBI bacterium ammonia GENESU (Brevibacterium ammoniagenes) ATCC6871, this ATCC13745, this ATCC13746; BUREBI bacterium DEBARIKATAMU () [Brevibacterium] divaricatumATCC14020; BUREBI bacterium RAKUTO fur mentum ATCC13869 (Brevibacteriumlactofermentum); Corynebacterium guru TAMIKAMU () [Corynebacterium] glutamicumATCC31831 grade can also be used as a host microorganism.

[0028] In addition, since it is the plasmid pBY502 (refer to JP,63-36787,A) which a bacteria stock holds when using the strain of the Brevibacterium flavum MJ-233 origin as a host and the transformation may be difficult, in such a case, it is desirable to remove a plasmid pBY502 from a bacteria stock. It is also possible to carry out a deletion to nature by repeating subculture as a method of removing such a plasmid pBY502, for example, and removing artificially is also possible [Bact.Rev., 36, and p.361 - 405 (1972) reference]. It will be as follows if an example of a method which removes the above-mentioned plasmid pBY502 artificially is shown.

[0029] It cultivates at about 35 degrees C for about 24 hours, checking [carry out inoculation to the culture medium containing an acridine orange (concentration : 0.2-50microg/(ml)) or an ethidium bromide (concentration : 0.2-50microg/(ml)) of concentration etc. which checks growth of host Brevibacterium flavum MJ-233 imperfectly so that it may become about 10 cells per ml, and] growth imperfectly. Culture medium is applied to the agar medium after dilution, and it will cultivate at about 35 degrees C for abbreviation two days. Plasmid extract operation is respectively performed independently from the colony which appeared, and the stock from which the plasmid

pBY502 is removed is chosen. The Brevibacterium flavum MJ-233 origin strain from which the plasmid pBY502 was removed by this operation is obtained.
[0030] The transformation by the aforementioned recombination plasmid of the above-mentioned host microorganism The method of itself known, For example, Calvin, N.M.and Hanawalt, P.C., Journal of Bacteriology, 170, 2796(1988);Ito, K., Nishida, T.and Izaki.K. and Agricultural and Biological By the method of a publication in reference, such as Chemistry and 52,293 (1988) For example, it can carry out by energizing a pulse wave to a host microorganism [Satoh, Y.et al., Journalof Industrial Microbiology, and 5,159 (1990) references].
[0031] The cultivation method of of the coryneform group of bacteria which has the aceto hydroxy-acid iso melodrama Jupiter-VIII KUTAZE production ability obtained by carrying out a transformation by the above-mentioned method, for example, a Brevibacterium flavum MJ-233 origin stock, is described below. cultivation can be performed by the usual nutrition culture medium including a carbon source, a nitrogen source, mineral salt, etc., and it is independent [ammonia, an ammonium sulfate, an ammonium chloride, an ammonium nitrate, a urea etc.] respectively as a glucose, ethanol, a methanol, blackstrap molasses, etc. and a nitrogen source as a carbon source, for example -- or it is mixed and used Moreover, as mineral salt, a phosphoric-acid 1 hydrogen potassium, a potassium dihydrogenphosphate, magnesium sulfate, etc. are used, for example. In addition, nutrients, such as various vitamins, such as a peptone, a meat extract, a yeast extract, corn steep liquor, a KAZAMINO acid, and a biotin, can be added to a culture medium.
[0032] Cultivation can usually be preferably performed about 20 - 40 degrees C of abbreviation to the bottom of aerobic conditions, such as aeration churning and shake, at the temperature of about 25 degrees C - about 35 degrees C. pH in the middle of cultivation -- 5-10 -- it can consider as the seven to 8 neighborhood preferably, and pH adjustment under cultivation can be performed by adding an acid or alkali the carbon source concentration at the time of a cultivation start -- desirable -- one to 5 capacity % -- it is two to 3 capacity % still more preferably Moreover, incubation period can usually be made into one - seven days, and the highest period is for three days.
[0033] Thus, biomasses can be respectively collected from the culture obtained, water or the suitable buffer solution can wash, and it can be used for L-isoleucine or L-valine generation reaction. In L-isoleucine or L-valine generation reaction, it can fix and use for suitable support as the crude enzyme separated from the biomass debris or it which could use these biomasses as they were, or added ultrasonication etc., or a refining enzyme. The debris of the **** biomass stated above and rough ****** call a refining enzyme, a fixed object, etc. a "biomass processing object" collectively on these specifications.
[0034] If a deer is carried out and this invention is followed, the manufacturing method of the L-isoleucine characterized by carrying out an enzyme reaction in the aquosity reaction mixture which contains a carbon source and a nitrogen source at least, and making L-isoleucine or L-valine generate under existence of the above-mentioned culture object or a biomass processing object, or L-valine will be offered. The above-mentioned enzyme reaction can usually be preferably performed about 20 - 40 degrees C of abbreviation within the limits of about 25 - 35 degrees C of abbreviation.
[0035] The carbon source which can be added in aquosity reaction mixture, and a nitrogen source can mention what is used for said usual nutrition culture medium. Moreover, in this

aquosity reaction mixture, the mineral salt which can be used for said usual nutrition culture medium can also be added. Especially when the host microorganism which can carry out a transformation by the plasmid of this invention is the coryneform group of bacteria of biotin demand nature, it is suitable to carry out an enzyme reaction and to make L-isoleucine or L-valine generate in the aquosity reaction mixture which contains a carbon source and a nitrogen source at least with the culture object prepared like the above or its fixed object, and does not contain a biotin. In this case, the coryneform group of bacteria of biotin demand nature is made to react to a carbon source and a nitrogen source through the enzyme reaction accompanied by energy conjugate in the metabolic system which this biomass holds, without carrying out biomass proliferation in the aquosity reaction mixture which does not contain a biotin substantially, and L-isoleucine or L-valine is manufactured.

[0036] If a deer is carried out and this invention is followed, under existence of the (1) above-mentioned culture object or its fixed object The manufacturing method of the L-isoleucine characterized by carrying out the enzyme reaction of the butanoic acid derivative to ethanol at least in the aquosity reaction mixture which does not contain a biotin, and making L-isoleucine generate, (2) The manufacturing method of L-valine characterized by carrying out an enzyme reaction in the aquosity reaction mixture which contains a glucose at least, and making L-valine generate under existence of the above-mentioned culture object or its fixed object is offered.

[0037] Although the above-mentioned aquosity reaction mixture according to this invention can also be the buffer solutions, such as water which does not contain a biotin substantially, a phosphoric acid, or a tris hydrochloric acid, the synthetic medium which does not contain a biotin preferably is used. The solution in which the chemical structure which does not contain natural nutrient matters, such as a yeast extract, a peptone, and a course tape liquor, contains the known source of an inorganic nitrogen and/or a known inorganic substance is included by this synthetic medium. As a source of an inorganic nitrogen of the synthetic medium which can be used in this invention, ammonia, an ammonium chloride, an ammonium sulfate, an ammonium nitrate, ammonium phosphate, etc. can be illustrated, for example, and a phosphoric-acid 1 hydrogen potassium, a potassium dihydrogenphosphate, magnesium sulfate, a manganese sulfate, an iron sulfate, etc. can be illustrated as an inorganic substance, for example. These sources of an inorganic nitrogen and mineral salt are independent respectively, or two or more sorts can be mixed and they can be used.

[0038] :(NH4)2 SO4 2 g/l;KH2PO4 0.5 g/l;K2 HPO4 0.5 g/l;MgSO4 and 7H2 O which is as follows when an example of the synthetic medium used in the manufacturing method of the L-isoleucine according to this invention or L-valine is shown 0.5 g/l;FeSO4 and 7H2 O 20 ppm;MnSO 4-4 - 6H2 O pH 7.6 solution contained 20 ppm.

[0039] Although especially the amount of the culture object which is the above used in the L-isoleucine of this invention or L-valine manufacturing method, and was made and prepared, or the biomass processing object used is not restricted, generally it can be preferably used 1 to 50% (wt/vol) on the basis of the capacity of a culture medium by the concentration of 2 - 20% (wt/vol) of within the limits. Generally the enzyme reaction using the culture object or biomass processing object in the water reaction mixture which has composition as described above can usually be preferably performed at the temperature of about 30 - 40 degrees C of abbreviation about 20 - 50 degrees C of abbreviation for

abbreviation 10- about 72 hours.
[0040] In the manufacturing method of the L-isoleucine according to this invention, in the aquosity reaction mixture which does not contain the above-mentioned biotin, ethanol, a butanoic acid derivative, and a nitrogen source carry out an enzyme reaction, and L-isoleucine is generated.
[0041] the concentration of the ethanol in aquosity reaction mixture for L-isoleucine manufacture -- usually -- 0.5 to 40 capacity % -- it can consider as within the limits of 1 - 20 capacity % preferably As a butanoic acid derivative in aquosity reaction mixture, DL-alpha aminobutyric acid, alpha-keto butanoic acid, or those salts can be mentioned, for example. Although it is appropriate to usually use it by 0.1 - 20% (wt/vol) of density range, if the concentration in reaction mixture adds without always exceeding 0.3% (wt/vol) especially when using alpha-keto butanoic acid or its salt, the concentration of the butanoic acid derivative in aquosity reaction mixture can reduce generation of the norvaline which is a by-product, the yield of L-isoleucine can also raise it, and it can deal in it. Addition of the above-mentioned reaction substrate may be continuously performed, unless the above-mentioned concentration is exceeded, or you may perform it intermittently. As a salt of the above-mentioned butanoic acid derivative in which it is used for a reaction and deals, for example, alkaline-earth-metal salts; ammonium salts [, such as alkali-metal salts; calcium,], such as sodium and a potassium, etc. are mentioned, and sodium salt is suitable also in them.
[0042] Moreover, in the manufacturing method of L-valine according to this invention, in the aquosity reaction mixture which does not contain the above-mentioned biotin, a glucose and a nitrogen source carry out an enzyme reaction, and L-valine is generated. Glucose concentration in aquosity reaction mixture for L-valine manufacture can usually be made into 0.1 - 5.0% of the weight of within the limits. As for a glucose, it is desirable to add to aquosity reaction mixture continuously or intermittently so that it may be maintained by the concentration of account within the limits of reaction Nakagami.
[0043] Separation from the aquosity reaction mixture of the L-isoleucine manufactured in this way or L-valine and refining can be performed according to how to usually use known in itself, for example, can be performed, combining suitably methods, such as an ion-exchange-resin approach and a crystallization method.
[0044]
[Example] Although this invention has been explained above, the following example explains still more concretely.
Cloning of the DNA fragment (A fragment) containing the gene which carries out the code of the aceto hydroxy-acid iso melodrama reductase of the example 1 Brevibacterium flavum MJ-233 origin [0045] Extraction semisynthetic-medium A culture-medium [composition of all DNA of Brevibacterium flavum MJ-233 : (A) 2g of ureas, 2 SO4 7g, K2 HPO40.5g, KH2 PO4 0.5g, (HN4) MgSO4 0.5g, FeSO4 and 7H2 O6mg, MnSO4 4 - 6H2 O 6mg, 2.5g of yeast extracts, 5g of casamino acids, biotin 200microg, thiamin-hydrochloride 200microg, Brevibacterium flavum MJ-233 (FERM BP-1497) was cultivated to glucose 20g and 1l. [of distilled water]] 1l. till the second half of a logarithmic growth phase, and the biomass was brought together in it. 10mM(s) which contain a lysozyme in the concentration of 10mg/ml for the obtained biomass NaCl-20mM tris-buffers (pH 8.0)-1mM It suspended in 15ml of EDTA-2Na solutions. Next, Protease K was added so that the last concentration might become [ml] in 100microg /, and it was

kept warm at 37 degrees C for 1 hour. Furthermore, it added so that the last concentration might become 0.5%, and at 50 degrees C, it was kept warm for 6 hours and the bacteriolysis of the sodium dodecyl sulfate was carried out. After adding equivalent phenol / chloroform solution to this lysate and shaking gently for 10 minutes at a room temperature to it, centrifugal separation (for 5,000xg and 20 minutes, 10-12 degrees C) of the whole quantity was carried out, the supernatant-liquid fraction was isolated preparatively, and after adding sodium acetate so that it may be set to 0.3M, the ethanol of quantitas duplex was added slowly. DNA which exists between a water layer and an ethanol layer was wound by the glass rod, and it was air-dry after washing by ethanol 70%. It is 10mM tris-buffers (pH 7.5)-1mM to obtained DNA. EDTA and 5ml of 2Na solutions were added, and it put at 4 degrees C overnight, and used for future experiments.
[0046] (B) Using a restriction enzyme EcoRI and Bgl II 50units, at 37 degrees C, 90microl of all the DNA solutions of Brevibacterium flavum MJ-233 obtained by the invention above-mentioned (A) term of a recombinant was made to react for 1 hour, and carried out full decomposition. This EcoRI and Bgl II What cut the cloning vector pKK 223-3 (it markets from Pharmacia) by the restriction enzyme EcoRI, and carried out flush-end processing is mixed after flush-end processing to Decomposition DNA. 50mM tris buffers (pH 7.6), 10mM dithiothreitol, 1mM ATP, 10mM MgCl2 And T4 Each component of DNA ligase 1unit is added (the concentration of each component is the last concentration), and it was made to react for 15 hours and was made to join together at 4 degrees C.
[0047] (C) Selection of the selection above-mentioned gene of the plasmid containing the gene which carries out the code of the aceto hydroxy-acid iso melodrama reductase was performed using aforementioned Escherichia coli variant Escherichia coli ME 8315. The transformation of aforementioned Escherichia coli ME 8315 is carried out by the calcium chloride method (Journal of Molecular Biology, 53, 159, 1970) using the plasmid mixture obtained by the above-mentioned (B) term. Selective-medium [K2 HPO4 7g, KH2 PO4 2g containing ampicillin 50mg, (NH4) 2 SO4 1g, MgSO4 and 7H2 O The smear of 0.1g, glucose 20g, leucine 20mg, thiamine 1mg, and the 16g of the agars was carried out to 1l. of distilled water at dissolution].
[0048] Liquid culture of the growth stock on this culture medium was carried out by the conventional method, and when plasmid DNA was extracted, the restriction enzyme cut this plasmid and it investigated using agarose gel electrophoresis from culture medium, in addition to the DNA fragment of length 4.6kb of a plasmid pKK 223-3, the insertion DNA fragment of about 2.1 kb(s) was accepted in length. It was as the size of the number of restriction enzyme recognition sites of the DNA fragment of about 2.1 kb(s) and a cutting fragment having been shown in the aforementioned table 1 in the length when cutting with various kinds of limits. The restriction enzyme cutting spot map of this DNA fragment is shown in drawing 1 .
[0049] Moreover, the plasmid obtained above was cut by various restriction enzymes, and the size of a cutting fragment was measured. The result is shown in the following table 2.
[0050]
[Table 2]
Table 2 Plasmid pKK223-IR Restriction enzyme The number of recognition sites Size of the piece of cutting (kb) SalI 4 4.05 1.2 0.95 0.5 PvuII 2 4.2 2.5 EcoRV 1 6.7 [0051] The plasmid characterized by the above-mentioned restriction enzyme was named pKK223-IR. The size containing the gene which carries out the code of aceto hydroxy-acid iso

melodrama Jupiter-VIII KUTAZE by the above was able to obtain the DNA fragment (Bgl II-EcoRI fragment) of about 2.1 kb(s).

[0052] In the length containing the gene which carries out the code of aceto hydroxy-acid iso melodrama Jupiter-VIII KUTAZE obtained by the (D) term of the determination example 1 of the base sequence of the gene which carries out the code of example 2 aceto hydroxy-acid iso melodrama Jupiter-VIII KUTAZE, about the DNA fragment of about 2.1 kb(s) The base sequence Plasmids pUC118 and pUC119 By the dideoxy nucleotide enzymatic process (the dideoxy chain termination method) (5463 Sahger, F.et al., Proc.Nat.Acad.Sci.USA 74, 1977) to be used It determined according to the strategic map shown in drawing 2 .

[0053] From existence of the open reading frame in the base sequence, the gene which carries out the code of aceto hydroxy-acid iso melodrama Jupiter-VIII KUTAZE consisted of base pairs of 1014 which carries out the code of the 338 amino acid which has the base sequence shown in array number:1 of the after-mentioned array table.

[0054] It reproduced within the example 3 coryneform group of bacteria, and it is the plasmid of molecular weight Dalton of about ten mega separated from BUREBI bacterium suitor thio varnish IFO12144 (VERM BP-2515), and to JP,1-95785,A, the stable manufacture plasmid pBY503 of the creation (A) plasmid pBY503 of a plasmid vector pCRY30 was carried out like a publication, and was prepared.

[0055] 2g of semisynthetic-medium A culture-medium [ureas, 2 (NH4) SO4 7g, K2 HPO4 0.5g, KH2 PO4 0.5g, MgSO4 0.5g, FeSO4 and 7H2 O 6mg, MnSO 4-4 - 6H2 O 6mg, 2.5g of yeast extracts, 5g of casamino acids, BUREBI bacterium suitor thio varnish IFO12144 was cultivated to BICHION200microg, thiamin-hydrochloride 200microg, glucose 20g, and 1l. [of distilled water]] 1l. till the second half of a logarithmic growth phase, and the biomass was brought together in it. The obtained biomass was suspended in buffer-solution [25mM tris (hydroxymethyl) aminomethane, EDTA [of 10mM], and 50mM glucose] 20ml which contains a lysozyme in the concentration of 10mg/ml, and it was made to react at 37 degrees C for 1 hour. Alkali-SDS [0.2N NaOH and 1% (W/V) liquid SDS] 40ml was added to reaction mixture, and it mixed gently, and put for 15 minutes at the room temperature. Next, it put into iced water for 15 minutes after adding potassium acetate solution [5M Mixed liquor of 60ml [of potassium acetate solutions], 11.5ml [of acetic acids], and 28.5ml of distilled water] 30ml to this reaction mixture and mixing with it enough.

[0056] The bacteriolysis object whole quantity was moved to the centrifuging tube, it applied to the centrifugal separation of 15,000xg for 10 minutes at 4 degrees C, and supernatant liquor was obtained. After adding and suspending equivalent phenol-chloroform liquid (phenol : chloroform = 1:1 mixing liquid) in this, it moved to the centrifuging tube, and applied to the centrifugal separation of 15,000xg for 5 minutes under the room temperature, and water layers were collected. The ethanol of quantitas duplex was added to the water layer, and it applied after 1-hour gentle placement at -20 degrees C, and applied to the centrifugal separation of 15,000xg for 10 minutes at 4 degrees C, and sedimentation was collected.

[0057] Precipitation was dissolved in pH 8.0 in TE buffer-solution [tris 10mM and EDTA 1 mM;HCl after reduced pressure drying at manufacture]2ml. Cesium-chloride solution [liquid made to dissolve 170g of cesium chlorides in 100ml of TE buffer solutions of concentration 5 times] 15ml and 1ml of 10mg [/ml] ethidium star's picture solutions were

added to the solution, and density was doubled [ml] in 1.392g /. Centrifugal separation of 116,000xg was performed for this solution at 12 degrees C for 42 hours.
[0058] A plasmid pBY503 is found out by UV irradiation as a downward band within a centrifuging tube. By extracting this band from the side of a centrifuging tube with a syringe, the fractionation liquid containing a plasmid pBY503 was obtained. Subsequently, this fractionation liquid was processed 4 times by equivalent isoamyl alcohol, extraction removal of the ethidium star's picture was carried out, and it dialyzed to TE buffer solution after that. Thus, after adding 3M sodium acetate solution to the dialysing fluid containing the obtained plasmid pBY503 at last concentration 30mM, the amount ethanol of double precision was added and -20 degrees C was put for 1 hour. 50microg Covered this solution over the centrifugal separation of 15,000xg, DNA was made to sediment, and plastic MISUDO pBY503 was obtained.
[0059]
(B) 37 degrees C (5units) of restriction enzymes SalI were made to react to creation plasmid pHSG298(TAKARA SHUZO make) 0.5microg of a plasmid vector pCRY30 for 1 hour, and plasmid DNA was decomposed completely. The restriction enzyme XhoI (1unit) was made to react to 2microg of the plasmid pBY503 prepared by the aforementioned (A) term for 30 minutes at 37 degrees C, and partial decomposition of the plasmid DNA was carried out.
[0060] The component in the this deactivation solution in order to mix both plasmid DNA decomposition product and to inactivate a restriction enzyme, after heat-treating for 10 minutes at 65 degrees C is 50mM tris-buffers pH7.6 and 10mM respectively as the last concentration. MgCl2, 10mM dithiothreitol, 1mM ATP and T4 Each component was strengthened so that it might be set to DNA ligase 1unit, and it was kept warm at 16 degrees C for 15 hours. The transformation of the Escherichia coli jump-on-minus109 competent cell (TAKARA SHUZO make) was carried out using this solution.
[0061] The transformant A 30microg [/ml] (the last concentration) kanamycin, L culture medium (trypton 10g --) containing 100microg [/ml] (last concentration) IPTG(isopropyl-beta-D-thio galactopyranoside) 100microg/ml (the last concentration) X-gal (5-BUROMO-4-chloro-3-indolyl-beta-D-galactopyranoside) 5g of yeast extracts, NaCl By 5g and 1l. of distilled water, and pH 7.2, it cultivated at 37 degrees C for 24 hours, and was obtained as a growth stock. What has been grown among these growth stocks to the white colony was chosen, and the plasmid was respectively extracted by the alkali-SDS method [T.Maniatis, E.F.Fritsch, J.Sambrook, and "Molecular coloning" (1982) p90 - 91 reference].
[0062] Consequently, plasmid pHSG298-ori by which the fragment of about 4.0 kb(s) of the plasmid pBY503 origin was inserted in the SalI part of a plasmid pHSG298 was obtained. Next, cloning of the DNA fragment of about 2.1 kb(s) which process plasmid pBY503DNA obtained by the aforementioned (A) term by restriction enzymes KpnI and EcoRI, and are obtained was carried out to KpnI and the EcoRI part of the above-mentioned plasmid pHSG298-ori using the same method, and the plasmid vector pCRY30 was prepared.
[0063] Plasmid pKK223-IR obtained by creation of example 4 plasmid pCRY-IR, and the (C) term of the introductory example 1 to a coryneform group of bacteria A restriction enzyme BamHI is used for 5microg 5 unitses. Make it react at 37 degrees C for 1 hour, and decompose and EcoRI linker (it markets from TAKARA SHUZO) 1microl is mixed

with what carried out flush-end processing. 50mM tris buffers (pH 7.6), 10mM dithiothreitol, 1mM ATP, 10mM MgCl2 And T4 Each component of DNA ligase 1unit is added (the concentration of each component is the last concentration), and it was made to react for 15 hours and was made to join together at 12 degrees C.

[0064] It is a restriction enzyme EcoRI about this DNA. What was made to react for 1 hour and was decomposed at 37 degrees C using 3units, Plasmid pCRY30 obtained by the (B) term of an example 3 It is a restriction enzyme EcoRI about 1microg. 1unit is used. What was made to react for 1 hour and was decomposed at 37 degrees C is mixed. 50mM tris buffers (pH 7.6), 10mM dithiothreitol, 1mM ATP, 10mM MgCl2 And T4 Each component of DNA ligase 1unit is added (the concentration of each component is the last concentration), and it was made to react for 15 hours and was made to join together at 12 degrees C. According to the aforementioned method, the transformation of 8315 stocks of aforementioned Escherichia coli ME is carried out using this plasmid. Selective-medium [K2 HPO4 7g containing kanamycin 50microg/ml, KH2 PO4 2g, 2 (NH4) SO4 1g, MgSO4 and 7H2 O The smear of 0.1g, glucose 20g, leucine 20mg, thiamine 1mg, and the 16g of the agars was carried out to 1l. of distilled water at dissolution].

[0065] this -- a culture medium -- a top -- growth -- a stock -- a conventional method -- liquid culture -- carrying out -- culture medium -- plasmid DNA -- extracting -- this -- a plasmid -- a restriction enzyme -- cutting -- agarose gel electrophoresis -- using -- having investigated -- a place -- a plasmid -- pCRY -- 30 -- length -- 8.6 -- kb -- a DNA fragment -- adding -- a size -- 2.4 -- kb -- [-- 2.1 -- kb --; -- aceto -- a hydroxy acid -- iso -- a melodrama -- the reductase -- containing -- a fragment -- 0.3 -- kb;tac

[0066] The transformation of the prepared plasmid DNA was carried out to the coryneform group of bacteria like the above. The transformation was performed using the electric pulse method as follows. The Brevibacterium flavum MJ-233 (FERM BP-1497) plasmid pBY502 removal stock was cultivated by the 100ml aforementioned A culture medium till the early stages of a logarithmic growth, it added and the shaking culture of the penicillin G was carried out for further 2 hours so that it might be set to one unit / ml, biomasses were collected by centrifugal separation, and the 20ml solution for pulses (272mM Sucrose, 7mM KH2 PO4, 1mM MgCl2;pH7.4) washed the biomass. Furthermore, centrifugal separation of the biomass was carried out, they were collected, it suspended in the 5ml solution for pulses, 50micro of plasmid DNA solutions l obtained with the above was mixed with the 0.75ml cell, and it put for 20 minutes underwater. Using the gene pulsar (Biorad make), it was set as 2500 volts and 25microFD, and the pulse was put for 20 minutes into after [impression] ice. Inoculation was carried out to the aforementioned A agar medium which moves the whole quantity to the 3ml aforementioned A culture medium, and contains kanamycin 15microg/ml (the last concentration) after 1-hour cultivation at 30 degrees C, and it cultivated for two - three days at 30 degrees C. From the kanamycin resistant stock which appeared, the plasmid was obtained using the method of a publication in the aforementioned example 3 (A) term. This plasmid was cut by various restriction enzymes, and the size of a cutting fragment was measured. The result is shown in the following table 3.

[0067]

[Table 3]

Table 3 Plasmid pCRY30-IR Restriction enzyme The number of recognition sites The size of the piece of cutting (kb) EcoRI 2 8. 6 2.4 KpnI 1 11.0 BamHI 1 11.0 SmaI 2 6.2 4.8

SacI 2 8.4 2.6 XhoI 1 11.0 [0068] The plasmid characterized by the above-mentioned restriction enzyme was named pCRY30-IR. The restriction enzyme cutting spot map of this plasmid pCRY30-IR is shown in drawing 3 . In addition, Brevibacterium flavum MJ233-IR in which the transformation was carried out by plasmid pCRY30-IR is the Agency of Industrial Science and Technology of 1-1-3, Higashi, Tsukuba-shi, Ibaraki-ken.

[0069] To what poured distributively 100ml of A culture media of the stability above of example 5 plasmid pCRY30-IR to 500ml **********, and carried out sterilization processing for 15 minutes at 120 degrees C Inoculation of the transformant Brevibacterium flavum MJ233-IR obtained in the example 4 is carried out. After performing a shaking culture at 30 degrees C for 24 hours, 100ml of A culture media prepared similarly was poured distributively to 500ml **********, the subculture was carried out to what sterilized for 15 minutes at 120 degrees C so that it might become the rate of 50cells(es) per ml, and, similarly the shaking culture was performed to it at 30 degrees C for 24 hours. Next, centrifugal separation was carried out, the harvest was carried out, the constant-rate smear was carried out to the plate agar which prepared the kanamycin using A culture medium and additive-free A culture medium which were added at a 15microg [/ml] rate after washing a biomass, and the growth colony after cultivation was counted at 30 degrees C on the 1st.
[0070] Consequently, the colony grown to kanamycin addition and the additive-free culture medium checked the advanced stability of that it is the same number and growing all A culture-medium growth colonies to a kanamycin addition culture medium, i.e., this plasmid, further.
[0071]
[Layout Table]
array number: -- length [of one array]: -- mold [of 1017 arrays]: -- number [of nucleic-acid chains]: -- double strand topology: -- kind [of straight chain-like array]: -- Genomic DNA origin organism name: -- BUREBI bacterium FURABAMU strain name: -- sign:peptide existence position: showing the feature feature of MJ233 array -- method:E
[0072] which determined 1 -1017 feature
Array ATG Greenwich civil time ATT GAA CTG CTT TAT GAT Greenwich civil time GAC Greenwich civil time GAC CTC TCC TTG ATC 48Met Ala Ile Glu Leu Leu Tyr Asp Ala Asp Ala Asp LeuSer Leu Ile 1 5 10 15CAG GGC CGT AAG glucose tolerance tests GCC ATC glucose tolerance test GGC TAC GGC TCC CAG GGC CAC GCA 96Gln Gly Arg Lys Val Ala Ile Val Gly Tyr Gly Ser Gln Gly His Ala 20 25 30CAC TCCCAG AAC CTCs CGCGAT TCT GGC glucose tolerance test GAGGTT GTC ATT GGT CTG 144 His Ser Gln Asn LeuArg Asp Ser Gly Val Glu Val Val Ile Gly Leu 35 40 45CGC GAG GGC TCC AAG TCC GCA GAG AAG GCA AAG GAA GCA GGC TTC GAA 192Arg Glu Gly Ser Lys SerAla Glu Lys Ala Lys Glu Ala Gly Phe Glu 50 55 60GTC AAG ACC ACC Greenwich civil times GAG Greenwich civil time GCA Greenwich civil time TGG Greenwich civil time GAC GTC ATC ATG CTC 240Val Lys Thr Thr Ala Glu Ala Ala Ala Trp Ala Asp Val Ile Met Leu 65 70 75 80CTG Greenwich civil time CCA GACACC TCC CAG GCA GAA ATC TTC ACC AAC GAC ATC GAG 288Leu Ala Pro Asp Thr Ser Gln Ala Glu Ile Phe Thr Asn Asp Ile Glu 85 90 95CCA AAC CTG AAC GCA GGC GAC GCA CTG CTG TTC GGC CAC GGC CTG AAC 336Pro Asn LeuAsn Ala Gly Asp Ala Leu Leu Phe Gly His Gly Leu Asn 100 105 110ATT CAC

TTC GAC CTG ATC AAG CCA Greenwich civil time GAC GAC ATC ATC glucose tolerance test GGC ATG 384Ile His Phe Asp Leu Ile Lys Pro Ala Asp Asp Ile Ile Val Gly Met 115 120 125glucose tolerance test GCG CCA AAG GGC CCA GGCCAC TTG glucose tolerance test CGC CGT CAG TTC glucose tolerance test GAT 432Val Ala Pro Lys Gly Pro Gly His Leu Val Arg Arg Gln Phe Val Asp 130 135 140GGC AAG GGT glucose tolerance test CCT TGC CTC ATC GCA GTC GAC CAG GAC CCA ACC GGA 480Gly Lys Gly Val Pro Cys Leu Ile Ala Val Asp Gln Asp Pro Thr Gly145 150 155 160ACC GCA CAG GCTCTG ACC CTG TCC TAC GCA GCA GCA ATC GGT GGC GCA 528Thr Ala Gln Ala Leu Thr Leu Ser Tyr Ala Ala Ala Ile Gly Gly Ala 165 170 175CGC GCA GGC glucose tolerance test ATC CCA ACC ACC TTC GAA Greenwich civil time GAG ACC GTC ACC GAC 576Arg Ala GlyVal Ile Pro Thr Thr Phe Glu Ala Glu Thr Val Thr Asp 180 185 190CTC TTC GGC GAG CAG Greenwich civil time glucose tolerance test CTC TGC GGT GGC ACC GAG GAA CTG GTC 624Leu Phe Gly Glu Gln Ala Val Leu Cys Gly Gly Thr Glu Glu Leu Val 195 200 205 AAG glucose tolerance test GGC TTC GAG glucose tolerance test CTCACC GAA Greenwich civil time GGC TAC GAG CCA GAG ATG 672Ly s-Val-Gly-Phe-Glu-Val Leu Thr Glu Ala Gly-Tyr-Glu-Pro-Glu Met 210 215 220GCA TAC TTC GAG GTT CTT-CAC-GAG-CTC-AAG CTC ATC GTT GAC CTC ATG 720Ala Tyr Phe Glu Val Leu His Glu-Leu-Lys-Leu-Ile Val Asp Leu Met225 230 235 240TTC GAA GGT GGC ATC AGC AAC ATG AAC TAC TCT glucose tolerance test TCT GAC ACC Greenwich civil time 768Phe Glu Gly Gly Ile Ser Asn Met Asn Tyr Ser Val Ser Asp Thr Ala 245 250 255 GAG TTC GGT GGC TACCTC TCC GGC CCA CGC GTC ATC GAT GCA GAC ACC 816Glu Phe Gly Gly Tyr Leu Ser Gly Pro Arg Val Ile Asp Ala Asp Thr 260 265 270AAG TCC CGC ATG AAG GAC ATC CTG ACC GAT ATC CAG GAC GGC ACC TTC 864Lys Ser Arg Met LysAsp Ile Leu Thr Asp Ile Gln Asp Gly Thr Phe 275 280 285ACC AAG CGC CTC ATC GCA AAC glucose tolerance test GAG AAC GGC AAC ACC GAG CTT GAG 912Thr Lys Arg Leu Ile Ala Asn Val Glu Asn Gly Asn Thr Glu Leu Glu 290 295 300GGT CTT CGT Greenwich civil time TCC TAC AAC AACCAC CCA ATC GAG GAG ACC GGC Greenwich civil time 960Gly Leu Arg Ala Ser Tyr Asn Asn His Pro Ile Glu Glu Thr Gly Ala305 310 315 320AAG CTC CGC GAC CTCs ATG AGC TGG GTC AAG glucose tolerance test GAC Greenwich civil time CGC GCA GAA 1008Lys Leu Arg Asp Leu Met Ser Trp Val Lys Val Asp Ala Arg Ala Glu 325 330 335ACC Greenwich civil time TAA 1017Thr Ala

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[Translation done.]